استخدام بعض مضادات الاكسدة لخفض سمية الاوكرا توكسين في الجرذان

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المستخلص

هدفت الدراسة الحالية الى تقليل سمية الاوكرا توكسين A باستخدام نوعين من مضادات الأكسدة وهي فيتامين E بتركيز 10 ملغم/كغم من وزن الجسم و مستخلص زهرة نبات الكجرات *Hibiscus subdariffa* بتركيزين هما 10و 20 ملغم/كغم من وزن الجسم على التوالي. استخدم خمس مجاميع من الجرذان الناضج من نوع Albino أعطيت المجموعة الأولى عليقه فقط وسميت بمجموعة السيطرة ، أما المجموعة الثانية فقد تم إعطائها سم الاوكرا A وقد أعطيت المجموعة الثالثة السم مع فيتامين E وأعطيت المجموعة الرابعة والخامسة السم مع مستخلص زهرة نبات الكجرات . تم تغذية هذه المجاميع الخمسة بعليقه خالية من مضادات الأكسدة وتم تجريع السم ومضادات الأكسدة فمويا ويوميا لمدة 30 يوم. تم تقييم كفاءة مضادات الأكسدة عن طريق قياس بعض المتغيرات الكيموحيوية في بلازما الجرذان. بالاضافة الى دراسة مقاطع لعينات انسجة الكلى والكبد والعضلات الهيكلية باستخدام المجهر الضوئي. أظهرت المجموعة التي عوملت بسم الاوكرا A فقط وبدون مضادات الاكسدة ارتفاعا في قيم Malonyldialdehyde، الكلوكوز ، الدهون الثلاثية ، النسبة التعصدية (A I) ، أنزيمات الكبد (Alanine Amino Transferase (ALT و Aspartate Amino Transferase (AST) وكذلك في الكرياتنين واليوريا ولكن سببت انخفاضا في مستوى الكولسترول. لم تظهر المجاميع الأخرى والتي عوملت بسم الاوكرا A مع مضادات الأكسدة تغيرات معنوية في قيم المتغيرات الكيموحيوية المذكورة أعلاه عند المقارنة مع مجموعة السيطرة. اظهر نموذج الكلى للمجموعة التي عوملت بالسم فقط دون إعطاء مضادات الأكسدة تلف في القنيات الملتوية الدانية ، وأظهرت عينة نسيج الكبد تلف في المنطقة المحيطية لفصيص الكبد ولم تظهر تغييرات في عينات العضلات الهيكلية. لم يلاحظ أي تغييرات في كل المجاميع التي بحثت في هذه الدراسة فيما يتعلق بعينات الكلى و الكبد.

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USE OF SOME ANTIOXIDANT TO MINIMIZE OCHRATOXIN A TOXICITY IN RATS

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ABSTRACT

The present study aimed to minimize ochratoxin A toxicity by use of oral administration of two types of antioxidants, first was α -tocopherol at a concentration of 10 mg/kg body weight and the second was the extract of *Hibiscus subdariffa* at two concentrations 10 mg/kg and 20 mg/kg body weight, respectively.Five groups of mature albino rats were used, group 1 was fed on a semi-synthetic feed without toxin, called control group , while group 2 was given ochratoxin A, group 3 was given ochratoxin A with α -tocopherol, group 4 and 5 was also given ochratoxin A with the extract of *Hibiscus subdariffa* .These groups were fed on a constant semi-synthetic feed free from antioxidants , with daily oral administration of ochratoxin A and antioxidants for a period of 30 days .The efficiency of the antioxidants were then evaluated by monitoring the changes in the serum values of some biochemical parameters. Moreover histopathological examination of tissue specimens obtained from the kidney, liver and skeletal muscle of the rats by the use of light microscope. The group that was treated only with ochratoxin A and without antioxidants showed elevations in the serum biochemical parameters, these were the Malonydialdehyde, glucose , triglycerides , atherogenic index (AI), liver enzymes (Aspartate Amino Transferase , Alanine Amino Transferase), creatinine and blood urea level, but caused a reduction in serum cholesterol level. Other groups which were treated with ochratoxin A and antioxidants showed changes in the above biochemical parameters that were insignificantly differed from that of the control group. The kidney specimens from Group 2 showed changes in proximal convoluted tubules

and the liver specimens of the same group showed involvement at the peripheral region of the hepatic lobule. Skeletal muscle specimens showed normal architecture .No changes were observed in all other groups investigated throughout the study regarding kidney and liver specimens, with normal anastomosing columns of hepatocytes that were having normal homogenous cytoplasm.

INTRODUCTION

Ochratoxins are secondary fungal metabolites of some toxigenic species of Aspergilli and penicilli (27). Based on current literature, the toxicity of ochratoxin A (OTA) may be the result of three major effects (7,25): Inhibition of ATP and protein synthesis and enhanced lipid peroxidation . Elimination or reduction of the ochratoxin – producing fungi in grains is important but it is not always successful, particularly during the pre-harvest period. In turn, control of established ochratoxicosis is of great importance and is a chief goal for many investigations (20,24). Antioxidants play an important role in animal's health by inactivating damaging free radicals produced through normal cellular activity and from various stresses (6). *Hibiscus sabdariffa* significantly reduced cholesterol content in blood and serum and successfully prevented oxidation of low-density lipoproteins in rats (43). The occurrence of OTA in food and feed is widespread, and had been shown to be hepatotoxic, nephrotoxic, teratogenic, and carcinogenic to single – stomach animals (24). Much attention has been focused on the antioxidative compounds present in edible plants because of some safety concerns in synthetic antioxidants. *H.sabdariffa* contains high concentrations of colorful purple flavonoids called anthocyanins that are anti-inflammatory. Anthocyanins are beneficial for skin and vascular health and also known to coat the surface of cell membranes and protect them from enzymatic and free radical damage (34,43). Oxidative damage may be one of the manifestation of cellular damage in the toxicity of ochratoxin A. Reactive oxygen species (ROS) have a major role in the mediation of cell damage.(31).

MATERIAL AND METHODS

The OTA producing fungus was isolated from corn and rice collected from Baghdad local markets and from fields of College of Agriculture - Baghdad University according to the direct plating methods of Mislive *et al.*(26) . Collected samples were stored in freezer at – 18C° for 72 h. befor analysis in order to destroy the mites and other insects which may interfere with analysis. Identification of Aspergilli species had been done by the classification keys Samson,*et al.*(33). Penicilli species were identified by comparison between the planted colonies on three culture media (Czapek's Agar, Malt Extract Agar, Czapek's yeast Extract Agar) and the photograph of the ideal colonies of the same species cultured on the same media with aid of the computer. Six isolates belong to *Aspegillus ochraceus* and ten isolates belong to Pnicilli species were cultured on the same media, then tested for OTA production capability Balzer *et al.* (4), the qualification of the isolated OTA was done by TLC (4). The OTA was produced from the isolate with the highest toxin production was given a cod as OTAM using rice as a substrate according to Trenk *et al.*(42). According to the method of Balzer *et al.*(4),ochratoxin A was extracted and purified by thin layer chromatography on silica gel using benzene and acetic acid (9:1) as a solvent. The amount of OTA was estimated in comparison with standard OTA using scanning densitometer(12). The extract of *H.subdariffa* flowers was prepared according to Duh and Yen, (11). A total of 25 mature albino rats weighted (65-95gm) were fed for 30 days at a constant semi-synthetic feed, antioxidant free (2). Rats were isolated in a relatively controlled environment at a temperature of about 25C° were divided into 5 groups (5 rats each). Group 1 were given daily orally 0.1 ml of 10% ethanol and considered as a control, group 2 were given daily 0.1 ml of 10% ethanol containing 10 μ g of toxin /kg of body weight ,group 3 were given daily 0.1 ml of 10% ethanol containing 10 μ g of toxin /kg of body weight and 0.1 ml seed oil containing 10 mg α - tocopherol. Group 4 were given daily with 0.1 ml of 10% ethanol

containing 10 μ g of toxin /kg of body weight and 0.1 ml water containing 10 mg *H.subdariffa* extract and group 5 were given daily 0.1 ml of 10% ethanol containing 10 μ g of toxin /kg of body weight and 0.1 ml water containing 20 mg *H.subdariffa* extract. At the end of the feeding study, blood samples were collected via a disposable plastic syringe from the heart of the rats .Serum total cholesterol (T.C) was measured by the cholesterol kit PAP100 ,using the enzymatic method (30), serum triglycerides were measured by triglyceride kit PAP 150, serum high density lipoprotein – cholesterol was measured by the HDLc kit PAP 150, using the enzymatic method (5), and serum LDL was determined indirectly using Friedewald equation (15). Serum total lipid peroxide determined according to Stocks and Dormandy (37) and serum glucose was determined by the glucose kit using enzymatic method (21). Serum Aspartate amino transferase was measured by the Aspartate amino transferase kit PAP 100 using the enzymatic method (22,29), and serum alanine amino transferase was measured by the alanine amino transferase kit PAP100 using the enzymatic method (22,29). Serum Urea was determined by the urea kit (Biocon, Germany). and serum creatinine was determined by the creatinine kit PAP 100 (Biomerieux, France Rats were killed at the end of this study by placing in a jar saturated with chloroform. After killing the animals were fixed on a cork plate, the abdomen was opened from the anterior abdominal wall. Part from liver, kidney and thigh muscle were taken; lengths of all specimens were 2 cm each. the samples were immersed Immediately in Bouin's fixative to prepare them for histological study. Tissue processing and staining technique was carried out according to David, (9).

RESULTS AND DISCUSSION

Concentration of OTA produced *Aspergillus ochraceus* and Penicilli spp. Table 1 showed difference in the ability of fungal isolates for OTA production. The highest level of OTA production was obtained from *A. ochraceus* , it reached 2400 μg/kg (isolate No.1), while the ability of Penicilli spp. to produce OTA were between 10-220 μg/kg.These differences may be related to genetic coding (13).able 2 shows that serum cholesterol level of group 2 rats was higher than that of groups 3, 4 and 5, but it is lower than that of the control group. This was due to feeding of control group on antioxidant free diet and that OTA reduced serum cholesterol (17), by inhibiting HMGCOA reductase receptors (1).

Table 1 Concentrations of OTA produced by various Aspergilli and Penicilli species isolated on corn and rice

Type of fungus	OTA conc. (μ g/kg)	Isolate number
Aspergillus ochraceus	2400	1
A. ochraceus	956	2
A. ochraceus	350	3
A. ochraceus	267	4
A. ochraceus	90	5
A. ochraceus	41	6
Penicillium citrinum	13	7
P. alicantinum	67	8
P. brevi-compactum	40	9
P. simplicissium	19	10
P. simplicissium	12	11
P. simplicissium	220	12
P. verrucosum	67	13
P. verrucosum	22	14
P. vainentinum	23	15
P. alicantinum	10	16

Table 2 Cholesterol , Triglyceride and Atherogenic index (AI) concentration in rats serum after 30 days of feeding

Groups	Cholesterol Conc. mg/dl	Triglyceride Conc.mg/dl	Atherogenic index (LDL/HDL)
1	110 a	59 b	1.53 b
2	79 b	150 a	3.50 a
3	51c	63 b	0.90 c
4	65c	67 b	0.88c
5	62c	64 b	0.68c

* Different letters within each column refer to significant difference between the means ($p<0.05$).*Data are represented as mean of 5 rats.

A significant increase in serum triglyceride concentration in group 2 rats. This is in consistent with the findings of Gentles *et al.* (17) who reported that OTA leads to elevation in serum triglyceride level and this also may be due to the effects of OTA on lipids metabolism as reported by Stremmel *et al.* (39).There was no significant difference between groups 3,4 and 5 when compared with the control group which may be due to the antioxidant effect. A significant increase in AI in group 2 rats was observed in comparison with control group. This may be due to the oxidative stress caused by OTA , that seems primary due to both increased plasma free radicals concentrations and a sharp reduction in antioxidant defense (23).The relative decreased in groups 3,4 and 5 serum AI in comparison with groups 1 and 2 serum AI , may be attributed to the activity of the extract in preventing the production of free radicals which ultimately leads to cellular oxidative stress. The elevation in the value

of group 1 serum AI in a comparison with groups 3, 4 and 5 serum AI may be due to the fact that group 1 was fed on antioxidant free diet.

Tablo 3 Total Malonyldialdohydo (MDA) , Glucoso concentration in rats serum after ?0 days of feeding

Group	MDA Conc. (mmol/L)	glucose conc. mg/dl
1	2.17 b	110 b
2	3.05 a	165 a
3	1.77 c	100 b
4	1.90 c	115 b
5	1.68 c	112 b

* Different letters within each column refer to the significant difference between the means ($p<0.05$).
*Data are represented as mean of 5 rats.

Table 3 shows a significant increased in group 2 serum conc. of MDA which is an end product of fatty acid peroxidation responsible for the cellular oxidative damage. The results of this work is compatible with Rizzo *et al.* (31) reports, that oxidative damage may be one of the manifestation of cellular damage in OTA toxicity. The OTA generates a reactive oxygen species(18). Reactive oxygen species have a major role in mediation of cell damage (31). Lipid peroxidation is considered as one of the manifestation of cellular damage in the toxicity of OTA (20). Groups 3 , 4 and 5 showed no significant difference in the level of the serum MDA and all were less than that of the control group. This was due to the fact that the control group was fed on antioxidant free diet, where antioxidants are considered to be as a natural lipophilic and hydrophilic that act as a defense factors by minimizing the production and action of the harmful oxidants (3).Group 5 showed a significant and considerable decrease in the level of serum MDA in comparison with group 1 and 2. This may be due to the high concentration (20mg) of the *H.subdariffa* extract which exhibited a more potent activity in minimizing the production and action of the harmful products., which indicate that lipid peroxidation and in consequence of better cellular protection against oxidative damage resulting from OTA. The results of this work are in compatible with reports of Sherwin (35) and Dziezak (10) who reported that antioxidants are believed to intercept the free-radical chain of oxidations and to contribute hydrogen from the phenolic hydroxyl groups themselves, thereby forming stable free radicals which do not initiate or propagate further oxidation of lipids. A significant increase in group 2 serum glucose level, which is in consistent with finding of Suzuki *et al.*(41) that blood samples from fasted treated rats with 100 μg OTA / rat by gavages for 8 weeks contained about twice the amount of glucose compered with controls. The diabetogenic effect of OTA

was thought to be due to inhibited synthesis or release of insulin from pancreatic cells, thereby suppressing glycolysis and glycogenesis and enhancing gluconeogenesis and glycogonlysis (40). The remaining groups showed no significant difference in the glucose level in comparison with that of the control group . This is due to that fact antioxidants act by inhibiting the generation of DNA adducts. At least some of the adducts might have been due to OTA-induced cytotoxic effects that generate reactive oxygen species (ROS). Thus Grosse *et al.* (18) found that prior treatment of rats with ascorbic or α-tocopherol significantly decreased the number of adducts. Reactive oxygen species ultimately lead to oxidative damage to DNA therein reducing pancreatic insulin synthesis (18 , 40) The primary effect of OTA is inhibition of protein synthesis ,thereby RNA and DNA synthesis may be inhibited (8) Table 4 shows that group 2 exhibited a considerable increase in serum creatinine and urea concentration ,this is in compatible with the finding of Farshid and Rajan, (14) Ramadeviet *et al.* (28) ; Saleem and Khafajii, (32) who reported that low levels of OTA in feed for few weeks caused increasing in the serum creatinine and serum urea. In groups 3 and 4 ،serum creatinine and urea concentration, were nearly equal to that at the control group. This finding was in agreement with what was reported by Chew (6), who mentioned that antioxidant play an important role in animals health by inactivating damaging free radicals produced through normal cellular activity and from various stresses. Group 5 showed a significant reduction in serum creatinine and urea concs., this may explain the finding that on higher conc. (20mg), the *H.subdariffa* extract is found to be more potent in cellular protection than when applying it at lower conc. (10mg).

Table 4 Creatinine and Urea concentration in rats serum after 30 days of feeding

Groups	Creatinine Conc.mg/dl	Urea Conc. mg/dl
1	0.96 b	35 b
2	1.8 a	54 a
3	0.98 b	33 b
4	0.96 b	31 b
5	0.5 c	18 c

*Different letters within each column refer to the significant difference between the means ($p<0.05$). *Data are represented as mean of 5 rats

Table 5 shows that a significant increase in group 2 serum AST and ALT conc. in a comparison with that of the control group. This is due to the fact that, OTA causes oxidative stress and production of free radicals in rat hepatocytes (16 , 20). Groups 3, 4 and 5 show no significant difference in serum AST and ALT in comparison with that of the control group may be due to the antioxidant activity

whereby reducing cellular damage through prevention of lipids oxidation. (20).

Table 5: Aspartate Amino Transferase and Alanine Amino Transferase concentration in rats serum after 30 days of feeding

Groups	AST Level i.u./L.	ALT Level i.u./L.
1	18.0 b	13.0 b
2	43.0 a	39.0 a
3	16.9 b	11.8 b
4	17.5 b	12.4 b
5	15.0 b	11.0 b

*Different letters within each column refer to the significant difference between the means ($p<0.05$). *Data are represented as mean of 5 rats

Histopathological examination

1. Kidney tissue

The proximal tubule is one of the main targets for nephrotoxic compounds such as OTA, which is characterized by degeneration of epithelial cells of the proximal tubules and interstitial fibrosis resulting in polyuria and various changes in haematological and biochemical parameters (38). Various groups of investigators have shown that this specific nephrotoxic effect is due to OTA induced defect in the organic anion transport mechanism located on the brush border of the proximal convoluted tubular cells and basolateral membranes (36). The organic ion transport system is also the mechanism by which OTA enters proximal tubular cells (36). Extensive kidney damage is indicated by the renal function test and by the sever histopathological changes. Examining the renal cortex showed normal configuration in cortical glomeruli in all the study group normal glomerulus with clear glomerular space and Bowmann's capsule (Fig. 1) The tubules in the renal cortex include proximal and distal convoluted tubules. In the group receiving toxin, cast (proteinaceous material) is seen filling the lumen with debris. The tubular epithelial cells are swollen, thus obliterating much of tubular lumen, and this findings, is in consistent with Stoev *et al.* (38) findings who was reported that the most striking histopathological changes seen in the kidney during Ochratoxicosis are swelling of the tubular epithelial cells, tubular dilatation and proteinaceous material (cast) in the lumen. The severity of these changes appeared parallel to the dose of OTA administered. There is also a differential effect on the convoluted tubules in that the proximal portion is more severely affected than the distal portion to the extent that some generalized necrosis of the epithelial cells of the proximal tubules is observed. Ochratoxin in rats differentially damages the proximal tubules , in the other groups, there is no cast and the cavity of the tubules is clearly visible. The tubular cells are normal in appearance (fig.2). This is due to the effect of the extract and vitamin E as antioxidants. Regarding the extract it's effect is attributed to the action of anthocyanins which are known to coat the surface of cell membranes and protect them from enzymatic and free radicals damage (34).

2. Liver tissue

The hypothesis that DNA damage induced by OTA is due to oxidative stress. Several experimental observations support this hypothesis. An unusually large number of DNA adducts is formed from OTA in low yields in various experimental systems (18). Some of these results are consistent with a major role of oxidative stress in the toxicity

of OTA. Antioxidants prevent the induction of DNA damage by OTA in mice (18). In group receiving the toxin, hepatic affection had been seen. Considering the hepatic lobule the involvement was in the peripheral region of the hepatic lobule. The region around the central vein was not affected and the middle region of the hepatic lobule was spared as well. The damage was involving the hepatocyte with no inflammatory cell infiltration was observed. (Fig.3). The hepatocytes showed cytoplasmic changes in form of cloudy cytoplasm. Cell boundaries between hepatocytes were not well distinct in the affected region. (fig. 4). Areas of disturbance of lobular architecture with evidence of hepatocytes necrosis were also seen. In other parts of the affected region, the changes were more advanced. There was degeneration of the cytoplasm in the form of vaculation and clumping however the cell integrity was not disturbed and no alteration in nucleus was seen. (Fig. 5). No change was observed in the liver in other groups under investigation, this is due to the antioxidant effect. In these groups hepatic cords were normal with normal anastomsing columns of cells (hepatoyces), which were having normal and homogenous cytoplasm and normal nucleus. (Fig. 6).

3. Muscle tissue

No change was observed in groups under study regarding skeletal muscles. Muscle fibers showed normal architecture. No fiber breaks down or changes in the sarcoplasm was observed. The myo-nuclei were peripheral in position. No central nucleation or nuclear changes were observed (Fig 7). This finding may be attributed to the low concentration of OTA used in this study, which on high concentration may cause muscle changes as Harwing *et al.*(19) reported that, the tissue distribution of OTA in pigs, rats, chickens and goats generally follow the order: Kidney > Liver > muscle > Adipose tissue. According to the mentioned results we conclude that vitamin E and extract of *H.subdariffa* flower had prevented the effect of OTA as being revealed in specimens of kidney and liver.

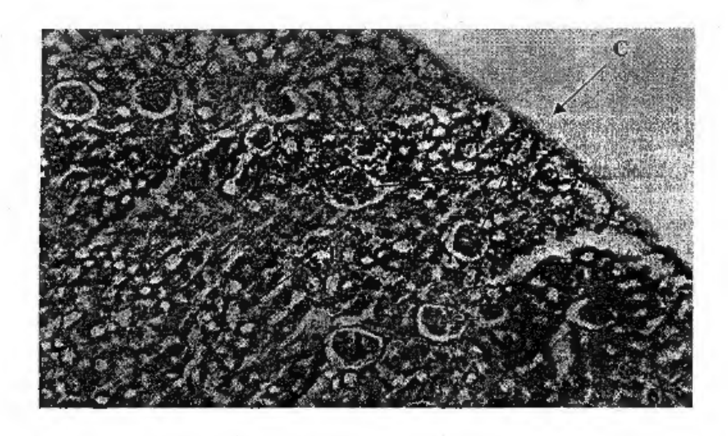
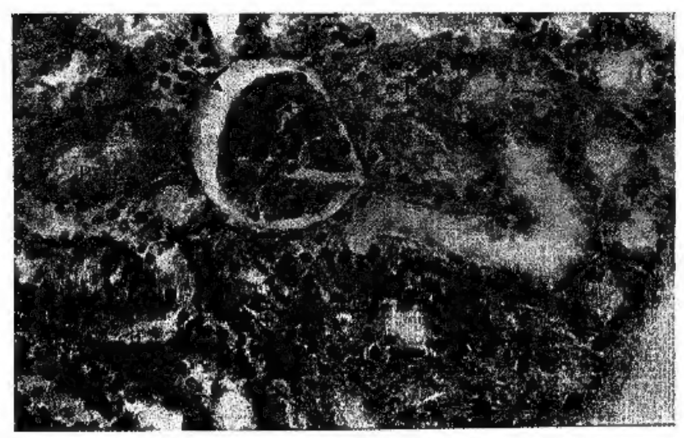


Fig 1:
A: Panoramic view of renal cortex.
C= capsule, G = Cortical glomeruli , T = Renal tubules.

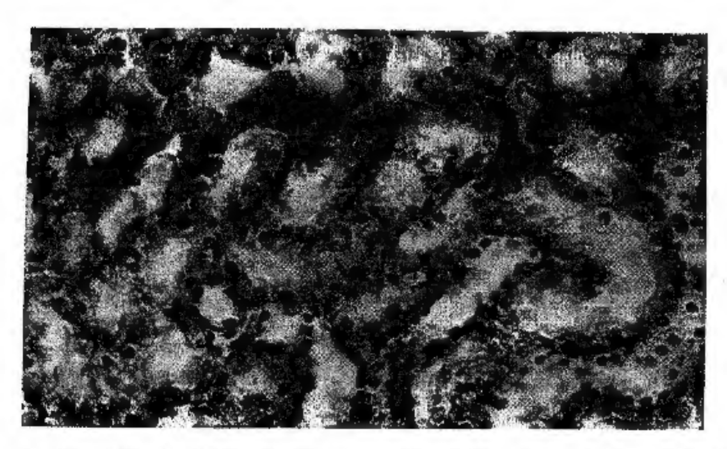
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B:Magnifying view of normal renal Cortical glomerulus
T=Glomerular capillary tuft., S=Glomerular space., B=Bowmann's capsule.
(Toxin- receiving group , haematoxylin and eosin staining 400 x).



Fig2
A:Renal tubules from toxin-receiving group showing cellular cast and obliteration of tubular lumen.



B: Renal tubules from other study group with clear tubular lumen and normal tubular lining cells with areas of necrosis.
(haematoxylin and eosin staining 400 X)

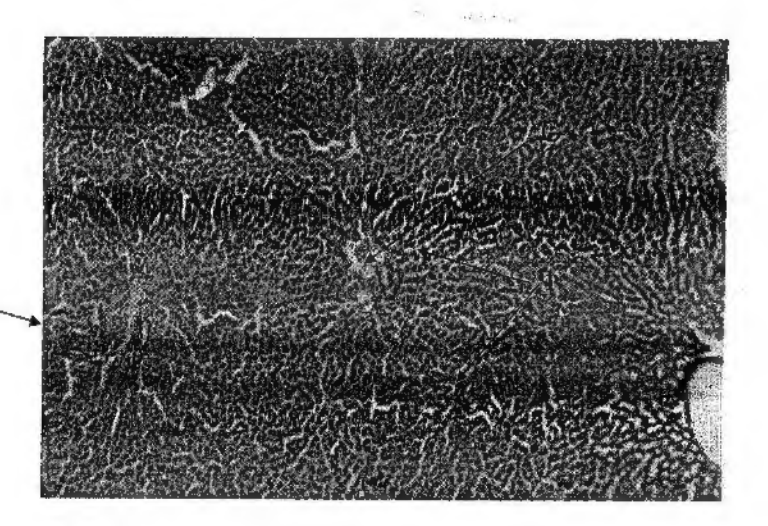


Fig. 3: Panoramic view of the liver
C= Central venule, T= Hepatic triade, P=Peripheral part of hepatic lobule
(toxin-receiving group. haematoxylin and eosin staining, 40 x)

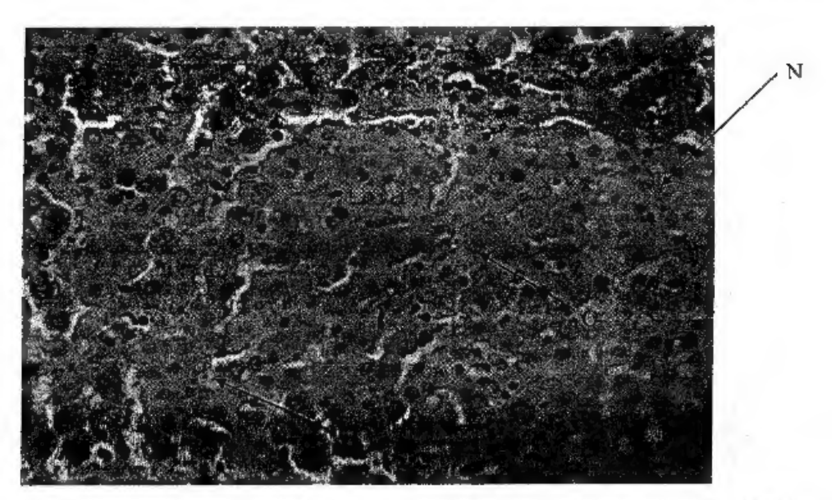
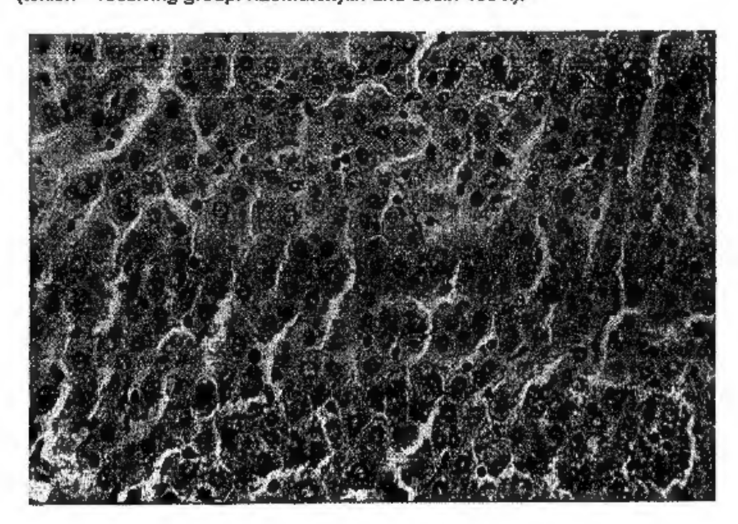


Fig. 4: Cords of hepatocytes from the peripheral part of the hepatic lobule. Cells show hazy cytoplasm and loss of distinction between adjacent cells with areas of necrosis.
S= Hepatic sinusoid, C= Cords of affected hepatocytes, N= Necrosis
(toxin-receiving group. haematoxylin and eosin staining, 400 X).

Fig. 5: Grossly involved hepatic lobule clumping of cytoplasm is seen. the nuclei are not affected with prominent nucleoli.
F=Fragmental clumped cytoplasm, N=Nucleus with prominent nucleolus.
(toxion – receiving group. haematoxylin and eosin 400 x).



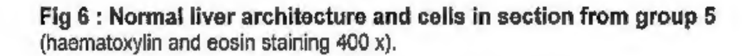


Fig 6 : Normal liver architecture and cells in section from group 5
(haematoxylin and eosin staining 400 x).



Fig 7 : Longitudinal section in thigh skeletal muscle fibers
F= muscle fibers, N= myo – nuclei
(toxin – receiving group. haematoxylin and eosin 400 x).

REFERENCES

1. Al-Gawari, A.A. 2000 Effect of Vitamin E on Cholesterol Level Reduction in Rats Blood.M.Sc.Thesis,Dept of Food Science and Biotechnology.Coll. of Agric., Univ of Baghdad, pp 85.

2. American Institute of Nutrition. 1977. Report of the American Institute of Nutrition Ad Hoc writing committee on standards for nutritional studies J . Nutr. 107 : 1340 – 1348 .

3. Atroshi, F; I. Biese, and H. Saloniemi. 2000. Significance of apoptosis and its relationship to antioxidants after OTA administration in mice. J Pharm Pharmacent Sci 3(3) ; 281-291.

4. Balzer, I.; B. Cedo and P. Stjepan. 1978. Rapid thin layer chromatographic method for determination Aflatoxin B1, Ochratoxin A and Zearalenone in corn. J. Ass. Off. Anal. Chem ., 61 : 584-585

5. Burstein, M. 1972. Lipid Res. 11, 583, cited by biomerieux kit manual, France.

6. Chew, B.P. 1995. Antioxidant vitamins affect food animal immunity and health . J. Nutri ., 125 : 18045 – 18085.

7. Creppy, E. E; R ,Röschenthaler and G .Dirheimer. 1984. Inhibitition of protein synthesis in mice by ochratoxin A and its prevention by phenylalanine. Food Chem., Toxicol. , 22: 883 – 886.

8. Creppy, E. E. ; G. Lorkowski; G. Beck; R. Röschenthaler and G .Dirheimer. 1980. Combined action of citrinin and ochratoxin A on hepatoma tissue culture cells. Toxicol. Lit ., 5, 375 – 380.

9. David, H. 1982. Fixation and Fixatives In:JD Bancroft and A Stevens. : Theory and Practice of Histological Techniques, 2nd edition., Churchill Livingstone, London p.20-26.

10. Diezak ,J.D . 1986. Antioxidants .Food Technol ., 40 :94-102.

11. Duh, P.D. and G.C Yen. 1997. Antioxidative activity of three herbal water extracts. Food Chemistry. 60 (2) : 639 – 645.

12. Eppley, R.M. 1968. Screening methods for zearolanone, aflatoxin, and ochratoxin. J. Assoc. Office. Agric. Chem. 51: 74- 78.

13. Fakhoury, A.M. and C.P.Woloshuk. 1999. Amyl, the α- amylase gene of *Aspergillus flavus* involvement in aflatoxin biosynesis in maize kernels. Phytopathology. 89 (10) : 908 – 914.

14. Farshid, A. and A .Rajan. 1995 Assessment of humoral immune response of Japanese quails in experimental ochratoxicosis Indian .Vet .J.,72(2): 122-125.

15. Friedwald, WT.and RI. Levy. 1972. Estimation of the concentration of LDLc in plasma without the use of preparative ultracentrifuge. Clinical Chemistry, 18:499.

16. Gautier, JC . 2001. Oxidative damage and stress response from Ochratoxin A exposures in rats. Radic. Biol. Med. 30 : 1089.

17. Gentles, A; E.E .Smith ; L.F. Kubena ; E. Duffus; P.Johnson ; J .Thompson; R.B. Harvey and T.S. Edrington. 1999. Toxicological evaluations ofcyclopiazonic acid and ochratoxin A in broilers . Poult Sci.,78 :1380 -1384 .

18. Grosse,Y; I. Chekir – Ghedira; A. Hue; S. Obrecht – Pflumio; G.Dirheimer; h .Bacha and A. Pfohl – Leszkowicz. 1997. Retinol, ascorbic acid and alpha – tocopherol prevent DNA adduct formation in mice treated with the mycotoxins ochratoxin A and zearalenone. Cancer Lett. , 114: 225 – 229.

19. Harwig, J; T. Kuiper-Goddman and P.M .Scott. 1983. Microbial Food Toxicants : Ochratoxin. In :M.Rechcigl. Handbook of Food Borne Diseases of Biological Origin, Boca Raton, FL : CRC Press, p. 193-238.

20. Hoehler, D .1997 Induction of free radicals in hepatocytes, mitochondria and microsomes of ochratoxin A and its analogs Biochem. Biophys. Acta 1357 : 225.

21. Kaplan L.A W.Ritschel and S.Kuo. 1984.Clin. Chem. The C.V.Mosby Co.St. Louis.Toronto.Princeton,p.1032-1036.

22. Karmen, A. 1955. The spectrometric assay of glutamic-oxalacetic transaminase in human blood serum. J. Clin . Invest. 34 : 131-133 .

23. Klein,R;Klein,BEK and SE.Moss. 1993.Prevalence of microalbuminuria in older-onset diabetes. Diabetes 16:1325-1330.

24. Kuiper – Goodman and D.L. Grant. 1993. Ochratoxin A. Toxicological Evaluation of Certain Food Additives and Contaminants, WHO Food Additives Series 28, IPCS, World Health Organization Geneva,p 208-254.

25. Marquardt, R. R and A. Frohilch. 1992. A review of recent advances in understanding ochratoxicosis. J. Anim. Sci . 70 : 3968 –3988.

26. Mislivec,P.B;C.T.Dieter and V.R.Bruce. 1975 .Mycotoxin producing potential of mold flora of dried beans. Appl.Microbiol.,29(4):522-526.

27. O'Callaghan, J ; M. X .Caddick and A. D.W. Dobson. 2003.A poly ketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceas*. Micriobiology. 149 : 3485 – 3491.

28. Ramadrvi, V; N. R. G.Nadu; and P.R.S. Raman. 1998. Pathology of broilers. Indian J. Vet . Pathol .,22 (2): 93-95.

29. Reitman, S. and S, Frankel. 1957 . A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Path. 28:56.

30. Richmond, W.1973.Preparation and properties of a cholesterol oxidase from nocardia species and its application to the

enzymatic assay of total cholesterol in serum .Clinical Chemistry, 19: 1350-1356.

31. Rizzo, AF; F. Atrshi; M. Ahotupa; S .Sankari and E .Elovaara. 1994. protective effect of antidants against free radical – mediated lipid peroxidation induced by Don or T-2 Toxin .J. Vet. Med. A 41 : 81 – 90.

32. Saleem, A.N. and N. J. Khlafaji. 2001. Intoxication due to mycotoxins extracted from feeding blocks and their treatment in rabbits. Iraqi .J. Vet . Sci ., 14 (1): 73-84.

33. Samson,R.A and E.S. van Reenen-Hoekstra. 1988 .Introduction to Food Borne Fungi .(3rd. Edn), Grafisch Netherlands,p.56-64.

34. Schmidgall, J; E.Schnetz and A.Hensel. 2000. Evidence for bioadhesive effects of polysaccharides and polysaccharide containing herbs in an ex vivo bioadhesion assay on buccol membranes. Plant Med. 66 (1) :48-53.

35. Sherwin, E.R.1978.Oxidation and antioxidants in fat and oil processing .J. Am .Oil Chem. Soc .,55 : 809-814 .

36. Sokol, P.P; G. Ripich; P.D. Holohan and C. R. Ross.1988. Mechanism of ochratoxin A transport in kidney .J. Pharmacol. Exp. Ther. , 246: 460 – 465.

37. Stocks, J and T.L. Dormandy .1971.the autoxidation of human red cell lipids induced by hydrogen peroxide. Brit. J. Hematol. 20 : 95-111.

38. Stoev,S.D ; S.Vitanov; G.Anguelov; T. Petkova - Bocharovaand E .E . Creppy. 2001. Experimental mycotoxic nephropathy in pigs provoked by a diet containing OTA and penicillic acid. Vet.Res. Commun.,25(3):205-223.

39. Stremmel, W; L. Pohl; A. Ring and T. Hervmann. 2001.A new concept of cellular uptake and intracellular trafficking of long – chain fatty acids. Lipids 36(9) : 981-989.

40. Subramanian, S; A .Kanthasamy; N. Balasubramanian; N ,Sekar and S .Govindasamy. 1989. OTA toxicity on carbonhydrate metabolism in rats. Bull. Environ. Contam. Toxicol., 43: 180-184.

41. Suzuki, S; Y. Kozuka; T. Satoh and M. Yamazaki. 1975.Studies on the nephrotoxicity of ochratoxin A in rats. Toxicol. Appl. Pharmacol., 34: 479-490.

42. Trenk, H.L ; M. E. Butz and F.S .Chu.1971.Production of ochratoxins in different cereal products by *Aspergillus ochraceus*. Applied. Microbiology. , 21(6) : 1032-1035.

43. Wichtl, M. 1994. Hibisci Flos-Red-Sorrel flower (English translation by Norman Grainger Bisset). In: Herbal Drugs and Phyto-Pharma Cuticles Vaishali C. CRC press, Stuttgart, p. 266-267.